

317

### Amendments to the Specification

Please replace the paragraph at page 3, beginning at line 4 with the following:

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Various avirulent viruses have been used as vectors. The gene for hepatitis B surface antigen (HBsAg) has been introduced into a gene non-essential for vaccinia replication. The resulting recombinant virus has elicited an immune response to the hepatitis B virus in test animals. Additionally, researchers have used attenuated bacterial cells for expressing hepatitis B antigen for oral immunization. Importantly, when whole cell attenuated *Salmonella* expressing recombinant hepatitis antigen were fed to mice, anti-viral T and B cell immune responses were observed. These responses were generated after a single oral immunization with the bacterial cells resulting in high-titers of the antibody. See, e.g., "Expression of hepatitis B virus antigens in attenuated *Salmonella* for oral immunization," F. Schodel and H. Will, *Res. Microbiol.*, 141:831-837 (1990). Others have had similar success with oral administration routes for recombinant hepatitis antigens. See, e.g., M.D. Lubeck et al., "Immunogenicity and ~~efficacy~~ efficacy testing in chimpanzees of an oral hepatitis B vaccine based on live recombinant adenovirus," *Proc. Natl. Acad. Sci.* 86:6763-6767 (1989); S. Kuriyama, et al., "Enhancing effects of oral adjuvants on anti-HBs responses induced by hepatitis B vaccine," *Clin. Exp. Immunol.* 72:383-389 (1988).

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{ Please replace the paragraph at page 5, beginning at line 1 with the following: }

Recent advances in genetic engineering have provided the requisite tools to transform plants to contain foreign genes. Plants that contain the transgene in all cells can then be regenerated and can transfer the transgene to their offspring in a Mendelian fashion.<sup>4</sup> Both [ ~~monocotyledonous~~ monocotyledonous and ~~dicotyledonous~~ dicotyledonous ] plants have been stably transformed. For example, tobacco, potato and tomato plants are but a few of the ~~dicotyledonous~~ dicotyledonous plants which have been transformed by cloning a gene which encodes the expression of 5-enolpyruvyl-shikimate-3-phosphate synthase.<sup>5</sup>

Please replace the paragraph at page 17, beginning at line 30 with the following:

B<sub>3</sub> Since many edible plants used by humans for food or as components of animal feed are ~~dicotyledenous~~ dicotyledonous plants, it is preferred to employ dicotyledons in the present invention, although monocotyledon transformation is also applicable especially in the production of certain grains useful for animal feed.

[ Please replace the paragraph at page 18, beginning at line 18 with the following: ]

B<sub>4</sub> There are various methods of introducing foreign genes into both ~~monocotyledenous~~ monocotyledonous and ~~dicotyledenous~~ dicotyledonous plants.<sup>33,34</sup> The principle methods of causing stable integration of exogenous DNA into plant genomic DNA include the following approaches: 1) Agrobacterium - mediated gene transfer;<sup>35,36,37,53</sup> 2) direct DNA uptake,<sup>38</sup> including methods for direct uptake of DNA into protoplasts,<sup>8</sup> DNA uptake induced by brief electric shock of plant cells,<sup>41,42</sup> DNA injection into plant cells or tissues by particle bombardment,<sup>39,44-46</sup> by the use of micropipette systems,<sup>43,47,48</sup> or by the direct incubation of DNA with germinating pollen;<sup>40,49</sup> or 3) the use of plant virus as gene vectors.<sup>33,51</sup>

[ Please replace the paragraph at page 18, beginning at line 26 with the following: ]

The Agrobacterium system includes the use of plasmid vectors that contain defined DNA segments that integrate into the plant genomic DNA. Methods of inoculation of the plant tissue vary depending upon the plant species and the Agrobacterium delivery system. A widely used approach is the leaf disc procedure which can be performed with any tissue explant that provides a good source for initiation of whole plant differentiation.<sup>6</sup> The Agrobacterium system is especially viable in the creation of transgenic ~~dicotyledenous~~ dicotyledonous plants.

[ Please replace the paragraph at page 26, beginning at line 10 with the following: ]

B<sub>5</sub> A. tumefaciens was cultured in 50 milliliters (50 ml) of YEP (yeast extract-peptone broth)<sup>58</sup> containing two-tenths milligrams per milliliter (0.2 mg/ml) streptomycin until the optical density (O.D.) at 600 nanometers (nm) of the culture reaches about five tenths (0.5). The cells were then centrifuged at 2000 times gravity (2000XG) to obtain a bacterial cell pellet. The Agrobacterium pellet was resuspended in ten milliliters of ice cold one hundred fifty millimolar

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sodium chloride (150mM NaCl<sub>2</sub>). The cells were then centrifuged again at 2000XG and the resulting Agrobacterium pellet was resuspended in one milliliter (1 ml) of ice cold twenty millimolar calcium chloride (20mM CaCl<sub>2</sub>). Five-tenths microgram (0.5 µg) of plasmid pHB101 or plasmid pHB102 was added to two tenths milliliters (0.2 ml) of the calcium chloride suspension of A. tumefaciens cells in a one and five tenths milliliter (1.5 ml) microcentrifuge tube and incubated on ice for sixty minutes. The plasmid pHB101 or pHB102 DNA and A. tumefaciens cells mixture was frozen in liquid nitrogen for one minute, thawed in a twenty-eight degree Celsius (28°C) water bath, and then mixed with five volumes or 1 milliliter (1 ml) of YEP (yeast extract-peptone broth). The plasmid pHB101 or pHB102 and A. tumefaciens mixture was then incubated at twenty-eight degrees Celsius (28°C) for four hours with gentle shaking. The mixture was plated on YEP (yeast extract-peptone broth) agar medium containing fifty micrograms per milliliter (50 µg/ml) kanamycin. Optimum drug concentration may differ depending upon the Agrobacterium strain in other experiments. The plates were incubated for three days at twenty-eight degrees Celsius (28°C) before selection of resultant colonies which contained the transformed Agrobacterium harboring the pHB101 or the pHB102 plasmids. These colonies were then transferred to five ~~millileters~~ milliliters (5 ml) of YEP (yeast extract-peptone broth) containing fifty micrograms per milliliter (50 µg/ml) of kanamycin for three days at twenty-eight degrees Celsius (28°C).

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[Please replace the paragraph at page 27, beginning at line 27 with the following:]

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The regenerated kanamycin-resistant pHB101 and pHB102 transformed tobacco plants were analyzed by hybridizing RNA samples with a <sup>32</sup>P ~~labelled~~ labeled probe encompassing the HBsAg gene coding region.

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[Please replace the paragraph at page 28, beginning at line 12 with the following:]

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Five micrograms of each RNA sample was denatured by incubation for fifteen minutes at sixty-five degrees Celsius (65°C) in twenty millimolar (20mM) MOPS (3-N-morpholino) propanesulfuric acid, pH 7.0; ten millimolar (10 mM) sodium acetate; one millimolar ethylenediaminetetraacetic acid (1 mM EDTA); six and one half percent (6.5% w/v)

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formaldehyde; fifty percent (50% v/v) formamide, and then fractionated by electrophoresis in one percent (1%) agarose gels. The nucleic acids were transferred to a nylon membrane by capillary blotting<sup>59</sup> for sixteen hours in twenty-five millimolar (25 mM) sodium phosphate, pH 6.5. Then the nucleic acids were crosslinked to the membrane by irradiation with ~~ultraviolet~~ ultraviolet (UV) light and the membrane pretreated with hybridization buffer [twenty-five hundredths molar (.25M) sodium phosphate, pH 7.0; one millimolar ethylene diamine tetraacetic acid (1mM EDTA); seven percent (7%) sodium dodecyl sulfate (SDS)] for one hour at sixty-eight degrees Celsius (68°C). The membrane was probed with 10<sup>6</sup> counts per minute per milliliter (cpm/ml) <sup>32</sup>P-labelled labeled random-primed DNA using a 700 base pair (bp) Bam HI-Acc I fragment from plasmid pKS-HBS which includes most of the coding region for HBsAg. Blots were hybridized at sixty-eight degrees Celsius (68°C) in hybridization buffer and washed twice for five hundred and fifteen minutes with forty millimolar (40 mM) sodium phosphate, pH 7.0 per one millimolar ethylene diaminetetraacetic acid (1mM EDTA) per five percent sodium dodecyl sulfate (5% SDS) at sixty-eight degrees Celsius (68°C) and exposed to X-OMAT AR film for twenty hours.

[ Please replace the paragraph at page 31, beginning at line 17 with the following: ]

B<sub>8</sub>  
Tomato, Lycopersicon esculentum var. VFN8, was transformed as in Example II. B and C by the leaf disc method using Agrobacterium tumefaciens strain LBA4404 as a vector, McCormick et al., 1986.<sup>23</sup> A. tumefaciens cells harboring plasmid pHB102, constructed as in Example II. A.2, which carries the HBsAg coding region fused to the tobacco etch virus untranslated leader, Carrington & Freed, 1990,<sup>73</sup> and the cauliflower mosaic virus 35S promoter, were used to infect cotyledon explants from seven day old seedlings. The explants were not preconditioned on feeder plates, but infected directly upon cutting, and co-cultivated in the absence of selection for two days. Explants were then transferred to medium B, McCormick et al., 1986,<sup>23</sup> containing five-tenths milligrams per ~~milliliter~~ milliliter (0.5 mg/ml) carbenicillin and one-tenth milligram per milliliter (0.1 mg/ml) kanamycin for selection of transformed callus. Shoots were rooted in MS medium containing one-tenth milligram per milliliter (0.1 mg/ml) kanamycin but lacking hormones, and transplanted to soil and grown in a greenhouse.

[ Please replace the paragraph at page 31, beginning at line 29 with the following: ]

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Several independent kanamycin-resistant callus lines were obtained after Agrobacterium-mediated transformation of the tomato variety VFN8. One of these lines regenerated shoots with high ~~frequency~~ frequency and was rooted and grown in soil in the greenhouse. The tissues from these plants were used for the protein and RNA analyses.

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